Mitochondrial double-stranded RNA triggers antiviral signalling in humans

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Mitochondria are descendants of endosymbiotic bacteria and retain essential prokaryotic features such as a compact circular genome. Consequently, in mammals, the mitochondrial DNA (mtDNA) is subjected to bidirectional transcription that generates overlapping transcripts capable of forming long double-stranded RNA (dsRNA) structures[1,2]. Nevertheless, mitochondrial (mt) dsRNA has not been previously characterized *in vivo*. Here, we describe the presence of a highly unstable native mtdsRNA species at single cell level and identify key roles for the degradosome components, mitochondrial dsRNA helicase SUV3 and exoribonuclease PNPase in restricting mtdsRNA levels. Loss of either enzyme results in massive accumulation of mtdsRNA that escapes into the cytoplasm in a PNPase dependent manner. This engages an MDA5 driven antiviral signalling pathway that triggers a type I interferon response. Consistent with these data, patients carrying hypomorphic mutations in PNPT1 display mtdsRNA accumulation coupled with upregulation of interferon stimulated genes (ISGs) and other markers of immune activation. The localization of PNPase to both the mitochondrial inter-membrane space (IMS) and matrix suggests its dual role in preventing formation and release of mtdsRNA into the cytoplasm. This in turn prevents the activation of potent innate immune defence mechanisms evolved to protect eukaryotic cells against microbial and viral attack.

Bidirectional transcription of mtDNA is an extreme example of convergent transcription in mammalian cells owing to symmetrical synthesis of both the heavy (H) and the light (L) strands. Notably, nearly the entire L-strand transcript undergoes rapid RNA decay by the RNA degradosome[3]. This decay process likely prevents the formation of potentially deleterious mtdsRNA. Indeed, among different cellular compartments, mitochondrial RNA (mtRNA) is known to be especially immunogenic[4]. Cellular nucleic acid sensors must discriminate viral nucleic acids from the vast excess of often biochemically indistinguishable cellular RNA and DNA as part of the innate immune response[5]. To achieve this, nucleic acid metabolism is pivotal in suppressing immune responses to self-nucleic acids[6]. Recently, numerous pathways have been shown to suppress mtDNA sensing by preventing its escape into the cytoplasm[7,8]. We sought to determine whether mitochondria are also a source of dsRNA *in vivo*, and in so doing uncover a pathway that suppresses the formation of immunostimulatory mtdsRNA.

We employed a monoclonal antibody (J2) specific for dsRNA as a widely used tool to detect viral dsRNA in animals and plants[9]. As shown previously, HeLa cell infection with the positive-strand RNA virus, EMCV resulted in strong cytoplasmic dsRNA signals (Extended Data Fig. 1a,b)[9]. Interestingly, weaker immunofluorescence (IF) signals were also observed in uninfected HeLa cells suggesting the existence of cellular dsRNA. To further characterize these cellular IF signals, fixed cells were pre-treated with structure specific RNases. IF signals were sensitive to dsRNA specific RNase III but not ssRNA specific RNase T1 or TURBO DNase confirming the presence of dsRNA at a single cell level (Extended Data Fig. 1c,d). We then verified the specificity of J2 for dsRNA using ss or dsRNA in *in vitro* immunoprecipitation (IP) experiments (Extended Data Fig. 1e). We next performed a J2 IP based dsRNA-seq to identify selected cellular dsRNA (Fig. 1a). Notably the mitochondrial genome generates nearly all detectable cellular dsRNA with 99% of the reads attributable to the mitochondrial genome (Extended Data Fig. 1f). Notably, the RNA-seq profile showed widespread reads from both H and L-strand of mtDNA, implying the presence of intermolecular dsRNA (Fig. 1b). This was confirmed by IF as 95% of J2 foci colocalized with mitochondria (Fig. 1c). To rule out potential artefacts caused by expression of mitochondrial pseudogenes integrated in the nuclear genome, we performed dsRNA staining in mtDNA depleted HeLa cells obtained by either expressing the Herpes Simplex Virus 1 (HSV-1) protein UL12.5M185 or human uracil-N-glycosylase (mUNG1)[10]. A lack of J2 signal confirmed that the dsRNA identified in our experiments can be wholly attributed to the mitochondrial genome (Extended Data Fig. 1g).

As dsRNA levels are normally suppressed in the cell, presumably to avoid the induction of an interferon response, we investigated mtdsRNA turnover. Actinomycin D (Act-D) treatment, which inhibits

mitochondrial transcription, caused a rapid loss of mtdsRNA, unlike the CDK9 inhibitor DRB which inhibits nuclear RNAPII transcription (Extended Data Fig. 2a). To search for factors involved in mtdsRNA suppression, we focused on the SUV3 and PNPase enzymes, known to be involved in degradation of L-strand transcripts[3]. siRNA-mediated depletion of either enzyme resulted in a 5-8 fold increase in dsRNA levels, based on both confocal microscopy (Fig. 1d,e,f) and flow cytometry (Extended Data Fig. 2b). The same effect was observed with a different set of siRNAs (Extended Data Fig. 2c). Other tested factors involved in mitochondrial nucleic acids metabolism had no effect on dsRNA levels (Extended Data Fig. 2d). We next confirmed that this increase in steady state levels of dsRNA was due to changes in mtdsRNA turnover. Upon Act-D treatment but not DRB, dsRNA levels in siCntrl treated cells were rapidly turned over (half-life of 30 min) whilst dsRNA levels were relatively stable for up to 3 hours in either SUV3 or PNPase depleted cells (Extended Data Fig. 2e).

To further understand the mechanism of dsRNA turnover by SUV3 and PNPase, we employed their catalytic mutants. Overexpression of a SUV3 transgene carrying an inactivating mutation (p.G207V) in the Walker A motif of the helicase into 293 cells acted as a dominant negative protein resulting in dsRNA accumulation (Extended Data Fig. 3a)[11]. Furthermore, northern blot analysis of J2 IP dsRNA isolated from this dominant negative mutant showed the accumulation of long dsRNA species, ~1-6kb, mapping over the entire mitochondrial genome (Extended Data Fig. 3b). Both RNA import and RNA turnover functions have been ascribed to PNPase[3,12]. Therefore, an R445E/R446E mutant of PNPase was employed that lacks exonuclease activity without affecting RNA import (Extended Data Fig. 4a)[3,12]. dsRNA levels accumulating upon PNPase depletion were suppressed by overexpression of siRNA resistant PNPase but not the R445E/R446E mutant in HeLa cells (Extended Data Fig. 4b,c,d) and 293 cells (data not shown). Overall, these results implicate the unwinding activity of SUV3 and the exonuclease activity of PNPase in dsRNA turnover. Consistently, J2 IP dsRNA-seq of SUV3 and PNPase depleted HeLa cells showed substantial accumulation of mtdsRNA as compared to siCntrl which were highly reproducible (Extended Data Fig. 5a,b).

Since long dsRNA is a hallmark of viral replication that triggers a type I IFN response, IFN-β induction was tested in various knockdowns of mitochondrial RNA processing factors. qRT-PCR analysis revealed a ~90 fold induction of IFN-β mRNA upon PNPase depletion but not SUV3 or MRPP1 (Fig. 2a). Consistently, gene expression profiling revealed activation of ISGs such as genes with direct antiviral activity (e.g. IFI44, IFIT1), cytoplasmic RNA sensors DDX58 and IFIH1 (encoding RIG-I and MDA5, respectively) and the transcription factor IRF7 that positively reinforces the antiviral response (Extended Data Fig. 6a). The realisation that mtdsRNA induced upon PNPase, but not SUV3, depletion

activated an IFN response suggested that SUV3 restricted mtdsRNA is either non-immunogenic or somehow concealed from cytosolic dsRNA sensors. We therefore isolated dsRNA from mitochondria depleted for SUV3 or PNPase using a MACS (magnetic-activated cell sorting) approach[13] and transfected this into HeLa cells to trigger IFN-β induction (Fig. 2b). Interestingly, mtRNA extracted from either condition triggered similar IFN-β induction which was RNase III sensitive (Fig. 2b). The latter finding confirms that the IFN induction is triggered by mtdsRNA and not by mtDNA. The experiment also excludes the possibility that SUV3 dependent mtdsRNA is non-immunogenic and led us to explore dsRNA localization. Transmission electron microscopy (TEM) immunogold labelling with J2 demonstrated mitochondrial localization of dsRNA in siCntrl samples that was substantially accumulated in SUV3 depleted cells (Fig. 2c). By contrast, in PNPase depleted cells, J2 staining displayed both a mitochondrial and cytoplasmic distribution, indicating the release of mtdsRNA into the cytoplasm (Fig. 2c). Consistently, enhanced mitochondrial outer membrane permeabilization (MOMP) of PNPase depleted cells using ABT-737 (Bcl-2 inhibitor) resulted in a ~3 fold induction of IFN-β levels (Extended Data Fig. 6b). Lack of an IFN response in SUV3 depleted cells with or without ABT-737 treatment suggested that mtdsRNA remains restricted to mitochondria (Extended Data Fig. 6b). We confirmed ABT-737 mediated MOMP through release of IMS localized protein cytochrome c into the cytoplasm (Extended Data Fig. 6c).

We wished to extend our results on PNPase restricted mtdsRNA in HeLa cells to an animal gene knockout (KO) model. We therefore employed the hepatocyte-specific *Pnpt1*^{HepKO} (HepKO) mouse that has a liver specific KO of PNPase as previously described[12]. We consistently observed an accumulation of dsRNA in PNPase HepKO liver sections versus control (Fig. 2d). Notably, PNPase KO cells showed a gradual loss of mtDNA over time, suggesting an adaptive response to IFN activation (Michael Teitell, unpublished results), and likely accounting for the heterogeneous increase in dsRNA levels (Fig. 2d, lower panel). However, differential gene expression analysis showed upregulation of *Ifnb1* and numerous ISGs like *If144*, *Ifit1*, and *Cxcl10* in PNPase HepKO mice (Fig. 2e, Extended Data Fig. 6d). These results are consistent with activation of a type I interferon response upon loss of PNPase and so support our HeLa cell siRNA depletion data in murine primary cells.

The importance of PNPase in restricting mtdsRNA led us to examine primary fibroblast cells from four different patients carrying biallelic hypomorphic mutations in the *PNPT1* gene (p.PNPase) identified by exome sequencing and clinical manifestation (Table 1 and Extended Data Table 1). These PNPT1 mutations led to decreased protein levels in fibroblasts of patients 2, 3 and to some extent 4, but not for the homozygous active site mutation p.Arg136His recorded in patient 1 (Fig. 3a)[14,15]. Fibroblasts

from all four patients demonstrated an accumulation of dsRNA (J2 signal) not observed in control cell (Fig. 3b). Moreover, this dsRNA colocalised with mitochondria (inset Fig. 3b).

Table 1 Summarized PNPT1 patient data

Patient	PNPT1 mutation	Amino acid change	Effect on PNPase	Outcome	IFN-α levels[#] in CSF	Neopterin* levels in CSF
1	c.407G>A hom	p.Arg136His	Abolishes active site	Died aged 2 years	NA	NA
2	c.208T>C c.2137G>T het	p.Ser70Pro p.Asp713Tyr	Reduced protein level	Alive at age 1 year	603	101
3	c.1495G>C c.1519G>T het	p.Gly499Arg p.Ala507Ser	Reduced protein level	Alive at age 7 years	ND	ND
4	c.1160A>G hom; ref[15]	p.Gln387Arg	Trimerization defective; reduced protein level	Alive at age13 years	ND	ND

CSF=Cerebrospinal fluid; [#]IFN-α (interferon alpha-normal range <1fg/l); *Neopterin normal range: 8-43 nmol/l, hom=homozygous; het=heterozygous; NA=not available; ND=not determined. See also Extended Data Table 1.

This was further established by enhanced accumulation of mtdsRNA (RNaseIII sensitive) in pure cytosolic fractions of patient 1 and 2 cells (Fig. 3c). Also, for three of the patients (no sample was available from patient 1), we recorded an upregulation of ISGs in peripheral blood (Fig. 3d). Furthermore, in patient 2, interferon-alpha protein measured by digital ELISA was elevated (603 fg/l) in cerebrospinal fluid (CSF), equivalent to levels observed in certain cases of viral meningitis[16] (Table1). Patient 2 also showed abnormally high levels of neopterin in the CSF (101 nmol/l), consistent with a hyper activated immune response[17] (Table 1). Overall, our analysis of patients harbouring hypomorphic mutations in PNPT1 clearly underlines the importance of preventing cytosolic sensing of mtdsRNA.

We sought to determine the mechanism of interferon activation by mtdsRNA in the context of PNPase deficiency. We tested the involvement of the RNA sensors RIG-I, MDA5 and TLR3 in this process. In PNPase-depleted HeLa cells, siRNA knock-down of MDA5, and to a minor extent of RIG-I but not of TLR3 abrogated the IFN response (Fig.4a). These data implicate MDA5 as the primary sensor of mtdsRNA. MDA5 signals via the mitochondrial antiviral signalling protein (MAVS) to induce type I IFNs so that MAVS knockdown also abrogated IFN-β induction (Fig. 4a, Extended Data Fig. 6e). We further confirmed these results by transfecting mtRNA isolated from PNPase-depleted HeLa cells (as in Fig. 2b) into RIG-I- (*RIG-I*$^{-/-}$) or MDA5-deficient (*MDA5*$^{-/-}$) murine embryonic fibroblasts (MEFs: Fig. 4b). *MDA5*$^{-/-}$, but not *RIG-I*$^{-/-}$cells, failed to upregulate mRNA levels of the ISG *Ifit1* in response to mtdsRNA. This strongly suggests that the mtdsRNA triggered interferon response is mediated through the

MDA5/MAVS axis. The possibility that mtdsRNA release into cytoplasm involves Bax/Bak, as in the case of mtDNA[7,18] was also investigated. Notably depletion of Bax/Bak prevented IFN-β mRNA induction following PNPase depletion suggesting that mtdsRNA release depends on Bax/Bak pores (Fig. 4c). As a final test for the escape of mtdsRNA into the cytoplasm following PNPase depletion, we tested for dsRNA editing by the adenosine deaminase ADAR1[19]. Notably 16 mitochondrial RNA editing sites (including 6 adenosines to inosines) were observed within the RNA-seq data of PNPase depleted cells compared to only one in SUV3 depleted sample (Extended Data Fig. 7a, b). Concurrent depletion of ADAR1 and PNPase enhanced the observed IFN response by 1.5 fold, suggesting that ADAR1 acts as a feedback suppressor of the innate immune response activated by mtdsRNA (Extended Data Fig. 7c, d). Overall, these mechanistic data on mtdsRNA formation, export and engagement with dsRNA sensors can be summarised in a model where the escape of mtdsRNA into the cytoplasm triggers an 'inappropriate' type I IFN response (Fig. 4d)

Our findings highlight an important function of PNPase which is underscored by its embryonic lethality in knockout mice and its identification as an essential fitness gene in CRISPR screens in human cell lines[12,20]. We considered it plausible that dysregulation of such an important pathway might induce an innate immune response, consistent with a disease class referred to as the type I interferonopathies[16]. Indeed, we show that biallelic hypomorphic mutations in PNPT1 cause mtdsRNA accumulation and immune activation. We suggest that mtdsRNA is a key mitochondrial-derived agonist of the innate immune system, a role until now mainly attributed to mtDNA[21]. Of note, genetic variants in MDA5 have been implicated in a number of human pathologies, both monogenic, and complex[22-27]. It is plausible that mtdsRNA mislocalization into the cytosol triggers an innate immune response upon viral infection as dsRNA accumulation is detectable upon viral infection of mammalian cells[9]. Interestingly, dsRNA accumulation upon EMCV infection in HeLa cells partially colocalizes with mitochondria (Extended Data Fig. 8). Possibly cellular mtdsRNA accumulation and its escape into the cytoplasm upon viral infection primes an antiviral response, as shown for mtDNA[8]. Overall, our results demonstrate a fundamental role of mitochondrial RNA processing in preventing the accumulation of deleterious self-nucleic acid (dsRNA) that would otherwise trigger innate immunity.

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Author Contributions

A.D. conceived the study, designed and performed most of the experiments and drafted the paper. S.D. performed all the bioinformatics analysis. L.S.B. with R.J.S. and A.D.Z. performed the experiments in Fig. 1d-f and Extended Data Figs. 1 (c, d, g), 2c-e, 3 and 4. L.J. and M.T. generated the IF and gene

expression data on PNPase HepKO. A.R., A.M. and M.S. provided patient fibroblasts and the clinical data. C.T. provided PNPase WB (Fig. 3a). Y.J.C. and G.I.R. generated the blood ISG expression data, and D.D. provided the IFN-α CSF data. T.N. provided Extended Data Fig.1e and C.R.A. performed FACS analysis. J.R. provided the MEF KO cells and agonists. A.D. discussed and interpreted results with inputs from N.J.P., R.J.S. and the other authors. N.J.P. and A.D. wrote the paper with input from the other authors.

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Figure Legends

Figure 1. Mitochondria form dsRNA, suppressed by the RNA degradosome.

a, dsRNA-seq experimental approach. **b**, dsRNA-seq reads across the mitochondrial genome spanning protein coding region in untreated HeLa cells. H-strand genes are shown as blue bars and L-strand as red bars. Short bars tRNA and long bars mRNA (representative of two experiments) **c,** immunostaining of HeLa cells with anti-dsRNA (J2). Mitochondria and nuclei stained with MitoTracker and Hoechst, respectively. Scale bars 10 µm. Graphs quantify co-localization of dsRNA foci with mitochondria. Mean ± SD, n=29 cells. **d**, anti-dsRNA (J2) staining in HeLa cells depleted for PNPase or SUV3 by siRNA as in c. Different imaging settings were applied in panel c and d so that control cell J2 intensity varies. **e**, western blot showing PNPase or SUV3 depletion (representative of four experiments). **f**, quantitation of dsRNA levels in PNPase or SUV3 depleted cells. Mean ± SD, n=4 cells. For gel source data, see Supplementary Figure 1.

Figure 2. PNPase suppresses a mtdsRNA mediated type I IFN response.

a, qRT-PCR analysis of IFN-β mRNA in HeLa cells treated with indicated siRNAs. Mean ± SD (n=3 independent experiments). **b**, boxed schematic representation of MACS strategy to purify mtRNA. Lower panel, qRT-PCR analysis of IFN-β mRNA in HeLa cells transfected with mtRNA (using MACS) as indicated. Mean ± SD (n=3 independent experiments) **c**, TEM images of immunogold labelled dsRNA in cryofixed HeLa cells treated with indicated siRNAs (representative of two experiments). M denotes mitochondria, scale 0.2 µM. **d**, fluorescent immunohistochemistry staining of dsRNA (J2) in liver sections from WT and *Pnpt1*^{HepKO} (HepKO) mice. Nuclei stained with DAPI. Lower panel shows scatter plots for dsRNA quantification. Mean ± s.e.m., n=41 WT and n=42 HepKO randomly sampled regions in 2 liver sections measured by 2-sided unpaired t test with Welch's correction. $^{**}P < 0.01$ **e**, log2 fold expression change of ISGs in HepKO vs WT female mice. ISG list is based on[8].

Figure 3. Pathological PNPT1 mutations result in mtdsRNA accumulation and activation of ISGs.

a, PNPase western blot in fibroblast from four patients with mutations in PNPT1 vs control. Quantitation is shown as average (n=4) (SD). **b,** dsRNA (J2) staining of PNPT1 patient-derived fibroblast cell lines vs control. Mitochondria were stained with MitoTracker. Scale bars 10 μm or 1 μm for zoomed-in inset (representative of two experiments). **c,** qRT-PCR analysis of cytosolic mtRNA (4 loci) in patient vs control cells (left panel). Mean ± SD (n=3 independent experiments). Fraction purity shown by western blots: P pellet and C cytosolic fractions (right panel) (representative of two experiments). **d**, qRT-PCR analysis of six ISGs in whole blood from patients 2, 3 and 4, ages in decimalized years when tested followed by interferon score in brackets. The data plotted is relative quantification (RQ) values for each patient, with the error bars representing RQmin and RQmax. n=29 healthy controls; n=3 independent patient samples. For gel source data, see Supplementary Figure 1.

Figure 4. MDA5 is the primary sensor of cytosolic mtdsRNA released in a Bax/Bak dependent fashion.

a, qRT-PCR analysis of IFN-β expression in HeLa cells transfected with indicated siRNAs. Mean ± SD (n=3 independent experiments). **b**, qRT-PCR analysis of *Ifit1* expression in *RIG-I*$^{+/-}$ (WT), *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ immortalised MEFs transfected with mtRNA, RIG-I (ppp-IVT-RNA^{99nt}) or MDA5 (CIP-EMCV RNA) specific agonists. Mean (n=2 independent experiments). Values are plotted on logarithmic scale. **c**, qRT-PCR analysis of IFN-β mRNA in HeLa cells treated with indicated siRNAs. Mean (n=2 independent experiments). Right panel shows western blot for siRNA depletion efficiency (representative of two experiments). For gel source data, see Supplementary Figure 1. **d**, model of mtdsRNA suppression by the RNA degradosome. Loss of PNPase causes mtdsRNA accumulation and release into cytoplasm in a Bax/Bak dependent manner. PNPase restricts mtdsRNA in matrix (together with SUV3) and IMS. MDA5 acts as the primary mtdsRNA sensor transducing an interferon response through the MAVS signalling pathway.

Methods

Antibodies and reagents

The following antibodies were obtained commercially: mouse anti-ADAR1 mAb (sc-73408) (Santa cruz), rabbit anti-PNPT1 (ab96176) (abcam), mouse anti-dsRNA mAb J2 (10010500) (Scions), anti-DNA (61014) (Progen), rabbit anti-SUV3(A303-055A)(Bethyl Laboratories), mouse anti-RIG-I mAb (Alme-1) (AG-20B-0009) (AdipoGen), rabbit anti-COX IV (3E11) (Cell signaling), rabbit anti-Cytochrome C (NB100-91732)(Novus Biologicals), rabbit anti-Calnexin (2433)(Cell signaling), mouse anti-Lamin A/C (4C11)(Cell signalling), donkey anti-mouse IgG (H+L) conjugated with Alexa Fluor 488 (A-21202) (Lifetech), goat anti-mouse IgM conjugated with Alexa Fluor 555 (A-21426, Thermo Fisher Scientific), normal mouse IgG2a (sc-3878), mouse anti-MDA5 (in house from J R), rabbit anti-MAVS (ALX-210-929-C100; Enzo lifesciences), rabbit anti-Bax (2772T; Cell signaling), rabbit anti-Bak (6947T; Cell signaling), rabbit anti-OXA1L (HPA003531; Sigma), mouse anti-α-Tubulin (T5168; Sigma), mouse anti-actin (ab8226;abcam), rabbit anti-FLAG (PA1-984B, Thermo Fisher Scientific), HRP secondary anti-mouse (ab6728;

abcam). ABT-737 (sc-207242) (santacruz), *5,6-dichloro-1-β-D-ribofuranosylbenzimidazole* (DRB) (sigma) or Act D (sigma), Digitonin (D141, sigma). ppp-IVT-RNA^{99nt} and CIP-EMCV-RNA were provided by Jan Rehwinkel[28].

Cell culture and siRNA transfection

HeLa, hSUV3_WT/hSUV3_G207V 293 cells[12], PNPase_WT/PNPase_R445E-R446E HeLa cells, or 293 Flp-In T-Rex cells (Thermo Fisher Scientific), MEFs (*RIG-I*$^{+/-}$, *RIG-I*$^{-/-}$, *MDA5*$^{-/-}$)[29] and skin fibroblasts were grown as a monolayer at 37°C, under 5% CO_2 in DMEM medium (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific). Skin fibroblasts were isolated from skin biopsies of controls and PNPT1 patients. Fibroblasts medium was supplemented with 2 mM L-glutamine, 2.5 mM pyruvate, 100 µg/ml streptomycin, 100 U/ml penicillin at 37°C. Silencing of genes of interest was performed using stealthRNA or other siRNAs (Extended Data Table 2) with Lipofectamine RNAiMAX (Thermo Fisher Scientific) in HeLa cells according to the manufacturer's instructions. The stealthRNA oligonucleotides and siRNAs were used at a final concentration of 20 nM. For double siRNA treatments, each siRNA was used at 20 nM final concentration. Cells were harvested three days after transfection or stated otherwise. For Flp-in cells, expression of exogenous genes was induced by addition of tetracycline to the culture medium at a concentration of 25 ng/mL.

Plasmid transfection and establishing of stable cell lines

Plasmid transfections were performed with TranIT2020 (Mirus) according to the manufacturer's instructions. HeLa cells 24h post transfection with plasmids encoding UL12.5M185 and mUNG1 were plated on glass coverslips and after one day of culturing were subjected to an immunofluorescence procedure as described in "Immunofluorescence" section. The stable, inducible cell lines were established using plasmids pRS946 (PNPase_WT), pRS950 (PNPase_R445E-R446E) and HeLa Flp-In T-Rex cells (kind gift from Matthias Hentze) as described previously[11]. The identity of HeLa Flp-In T-Rex cells was confirmed using STR profiling by DSMZ (Germany).

Mice

Hepatocyte-specific *Pnpt1*^{HepKO} (HepKO) mice were generated by breeding *Alb*$^{CRE/WT}$*/Pnpt1*$^{neo\text{-}flox/neo\text{-}flox}$ x *Alb*$^{WT/WT}$*/Pnpt1*$^{neo\text{-}flox/neo\text{-}flox}$ as described[12].

Identification of PNPT1 mutations

Exome sequencing was performed on genomic DNA (1 µg) isolated from blood leukocytes. Exons were captured by the in-solution enrichment methodology (SureSelect Human All Exon Kits Version 3, Agilent, Massy, France) using biotinylated oligonucleotide probe library (Human All Exon v3 50 Mb, Agilent). Each genomic DNA was then sequenced as paired-end 75 bases (Illumina HISEQ2000, Illumina, San Diego, USA). After demultiplexing, sequences were aligned to the reference human genome hg19 using the Burrows-Wheeler Aligner (version 0.7.12). Downstream processing was carried out with the Genome Analysis Toolkit (GATK 3.7), SAMtools (version 1.4), and Picard (version 2.9.0-1), following documented best practices

(http://www.broadinstitute.org/gatk/guide/topic?name=best-practices). Variant calls were made with the GATK Unified Genotyper. The annotation process was based on the latest release of the Ensembl database (version 75), dbsnp (version 140), 1000 genome project (version 2013/05/02), Gnomad (version 2.0.2) and EVS (version ESP6500SI-V2). Variants were annotated and analyzed using the Polyweb software interface designed by the Bioinformatics platform of University Paris Descartes. Sequences were filtered against SNPs (>0.1% frequency) reported in public (dbSNP, 1000 genomes and Exome Variant Server) and in-house databases including intergenic and non-coding region variants. Only homozygous variations were considered for patient 1, born to consanguineous parents, resulting in a list of 14 genes with only *PNPT1*, encoding a mitochondrial protein. Targeted exome sequencing using a panel of known genes for mitochondrial disorders was performed for patient 2 and two heterozygous *PNPT1* mutations were identified. Exome sequencing was performed for patient 3 and her non-consanguineous parents. The same filtering was used, identifying only one gene with two compound heterozygous mutations, *PNPT1*. DNA sequencing confirmed these mutations as well as their segregation with the disease in the families. All these variations were predicted to be deleterious by several softwares (Extended Data Table 1). Informed consent for diagnostic and research studies was obtained for all subjects in accordance with the Declaration of Helsinki protocols and approved by local Institutional Review Boards in Paris like human research participants ethics committee, Comité de Protection des Personnes, Ile de France II.

Targeted interferon stimulated gene (ISG) RNA expression in total blood

Whole blood was collected into PAXgene tubes, total RNA extracted using a PreAnalytix RNA isolation kit and RNA concentration assessed using a spectrophotometer (FLUOstar Omega, Labtech). Quantitative reverse transcription polymerase chain reaction (qPCR) analysis was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems), and cDNA derived from 40 ng total RNA. To generate a standard 6 probe interferon score, TaqMan probes for *IFI27* (Hs01086370_m1), IFI44L (Hs00199115_m1), *IFIT1* (Hs00356631_g1), *ISG15* (Hs00192713_m1), *RSAD2* (Hs01057264_m1) and *SIGLEC1* (Hs00988063_m1) were employed. The relative abundance of each target transcript was normalized to the expression level of *HPRT1* (Hs03929096_g1) and *18S* (Hs999999001_s1), and assessed with the Applied Biosystems StepOne Software v2.1 and DataAssist Software v.3.01. For all 6 probes, individual data were expressed relative to a single calibrator. RQ (relative quantification) is equal to 2^{-DDCt} i.e. the normalized fold change relative to the control data. The median fold change of the 6 genes compared to the median of 29 previously collected healthy controls was used to create an interferon score for each individual, with an abnormal interferon score being defined as greater than +2 standard deviations above the mean of the control group i.e. 2.466. Experiment was performed in triplicate from one blood sample obtained from each individual.

Quantification of interferon alpha (IFN-α) in cerebrospinal fluid (CSF) by Simoa assay

Simoa IFN-α assay was developed using a Quanterix Homebrew Simoa assay and two autoantibodies specific for IFN-α isolated and cloned from two APS1/APECED patients as recently described[16,30]. The 8H1 antibody clone was used as a capture antibody after coating on paramagnetic beads (0.3mg/mL), and the 12H5 was biotinylated

(biotin/Ab ratio = 30/1) and used as the detector. Recombinant IFN-α17/αI (PBL Assay Science) was used as a standard curve after cross-reactivity testing. The limits of detection (LOD) were calculated by the mean value of all blank runs + 3SDs and was 0.23 fg/mL.

Reverse transcription and real-time qPCR analysis

Total RNA was treated with TurboDNase (Ambion) and reverse-transcribed using SuperScript Reverse Transcriptase III (Invitrogen) with oligo $(dT)_{20}$ for IFN-β, *Ifit1*, L-mRNA (EMCV). Real-time quantitative PCR (qPCR) was performed with 2x Sensimix SYBR mastermix (Bioline) and analysed on a Corbett Research Rotor-Gene GG-3000 machine.

Immunofluorescence of HeLa cells infected with EMCV or transcription inhibitors and skin fibroblasts

HeLa cells were grown on a cover slip in a 6 well plate 24 h prior to treatment. HeLa cells were infected with EMCV at MOI 1 for indicated time point. For transcription inhibitor treatment, cells were treated with DMSO or Act-D or DRB at indicated concentrations for 60 min. For J2 IF on HeLa or skin fibroblasts, cells were incubated with Mitotracker CMXRos (100 nM) for 30 min at 37^0C before fixing in 4% PFA in PBS. Cells were washed thrice with PBS and permeabilized with 0.25% Triton X-100 in PBS. Cells were then washed with 0.05% Tween20-PBS and incubated with 3% BSA in PBS for 30 min at room temperature (RT). Primary antibodies anti-dsRNA (J2) were used at 1:200 in 3% BSA in PBS for 1 h at RT. Cells were washed thrice with 0.05% Tween20-PBS followed by incubation with secondary donkey anti-mouse IgG (H+L) conjugated with Alexa Fluor 488 at (1:300) concentration. Cells were then washed thrice with 0.05% Tween20-PBS and twice with PBS, and mounted with Vectashield mounting media with DAPI (Vector Laboratories). Z-stack images were collected with a FluoView1000 confocal microscope (Olympus) using a UPLSAPO 60.0X / 1.35 oil objective. Images were analyzed using ImageJ and prepared using OMERO software.

Immunofluorescence of nuclease treated samples

HeLa cells were plated on glass cover slips one day prior to fixation. Mitochondria specific dye MitoTracker DeepRed (200 nM) was added to culture 1 h prior to fixation. Cells were washed twice with PBS and fixed in 5% (v/v) formaldehyde, 0.25% (w/v) Triton X-100 and Hoechst 33342 (2 µg/mL) in PBS for 30 min in room temperature. Cells were washed thrice with PBS. Following enzymes were used: RNase T1 (EN0541, Thermo Fisher Scientific, concentration 100 U/mL), RNase III (M0245S, NEB, concentration 40 U/mL), TURBO DNase (AM2238, Thermo Fisher Scientific, concentration 40 U/mL). Enzymes were added in PBS containing 5 mM $MgCl_2$. Samples were incubated in 37°C for 30 min and washed thrice with PBS. Cells were incubated with 3% (w/v) BSA in PBS for 30 min. Primary antibodies anti-dsRNA (2.5 µg/ml) and anti-DNA (0.5 µg/ml) were used in 3% (w/v) BSA 8 h at 4°C. Cells were washed thrice with PBS and secondary goat IgG anti-mouse IgG2a conjugated with Alexa Fluor 488 and goat anti-mouse IgM conjugated with Alexa Fluor 555 (Thermo Fisher Scientific) were used at 2 µg/ml concentration in 3% (w/v) BSA. Cells were incubated for 1h at RT and washed thrice with PBS and mounted. Slides were imaged with a FluoView1000 confocal microscope (Olympus) and with ScanR fluorescence microscopy system (Olympus) (using UPlanSApo 20.0x objective) adapted for high throughput

image acquisition. The latter was used for quantitative fluorescent signal analysis. Quantitation was performed for at least 1755 cells per condition. Images were analyzed using ScanR_2.7.2 analysis software (Olympus). The same microscope instrument settings were used for all samples.

RNA polymerase inhibition

For Extended Data Figure 2e, HeLa cells were treated with siRNA for 3 days in 384-well format. Prior to fixation cells were treated for a given time with inhibitors of transcription: actinomycin D (0.5 µg/ml), DRB (100 µM). Detailed procedure is described in "siRNA transfection in 384-well format" and "Immunofluorescence labeling" Method section. Quantitative analysis of dsRNA fluorescent signal was performed with ScanR fluorescence microscopy system. This analysis was performed for at least 500 cells per replica per condition. Images were analyzed using ScanR_2.7.2 analysis software (Olympus).

siRNA transfection in 384-well format

Cells were reversably transfected in 384-well microplates (781946, Greiner Bio-One) using siRNA (final concentration 20 nM) and Lipofectamine RNAiMAX according to the manufacturer's instructions (Thermo Fisher Scientific). Cells were plated with Multidrop Combi Reagent Dispenser (Thermo Fisher Scientific). After 64 h, cells were subjected to an immunofluorescence procedure described in "Immunofluorescence labeling" Method section. Cells were left in PBS for imaging. All PBS washes were performed with 405 LS Microplate Washer (BioTek) and all other solution were added with Multidrop Combi Reagent Dispenser.

Immunofluorescence labelling

1 h prior to fixation mitochondria specific dye MitoTracker DeepRed (M22426, Thermo Fisher Scientific, 200 nM) was added to culture. Cells were washed twice with PBS and fixed for 30 min with PBS solution containing 5% (v/v) formaldehyde, 0.25% (w/v) Triton X-100 and Hoechst 33342 (2 µg/mL). Cells were washed thrice with PBS and incubated with 3% (w/v) BSA in PBS for 30 min. Primary antibodies anti-dsRNA, concentration: 2.5 µg/ml) were used in 3% (w/v) BSA over night at 4°C. Cells were washed thrice with PBS and secondary goat IgG anti-mouse IgG2a conjugated with Alexa Fluor 488 and goat anti-mouse IgM conjugated with Alexa Fluor 555 (Thermo Fisher Scientific) were used at 2 µg/ml concentration in3% (w/v) BSA. Cells were incubated 1h at RT and washed thrice with PBS. Cover slips were mounted with ProLong Gold Antifade Mountant (P36930, Thermo Fisher Scientific) or left in PBS if imaged with a ScanR fluorescence microscopy system. If the samples were subjected to quantitative analysis, the same microscope instrument settings were applied.

Co-localization of dsRNA with mitochondria

Cells were subjected to staining as described in "Immunofluorescence labelling" Method section. Z-stack images of microscopic slides were collected with a FluoView1000 confocal microscope (Olympus) using a PLANAPO 60.0 x 1.40 oil objective. XY optical resolution of images was 215 nm. Images were analyzed using Imaris_7.2.3 software (Bitplane). Object based colocalization of spots was performed. Colocalization of J2 spots with

mitochondria was based on fluorescence intensity from MitoTracker. Quantitation was performed for 29 randomly selected cells.

High-throughput fluorescence imaging

Data presented on Figure 1f, Extended Data Figure 2c, 2d and 4d were obtained using a ScanR fluorescence microscopy system (Olympus, UPlanSApo 20.0x objective). Images were analyzed using ScanR 2.7.2 analysis software (Olympus). Quantitation of fluorescent signal was performed for at least 400 cells per replica per condition.

Fluorescent Immunohistochemistry

Fluorescent IHC staining of 4 μm-thick formalin-fixed, paraffin-embedded (FFPE) tissue sections was performed on livers from WT C57BL/6 and *Pnpt1*^{HepKO} (HepKO) mice aged (6 weeks) and sex (female) matched littermates on a pure C57BL/6 background. FFPE slides were deparaffinized by immersion in 100% xylene, two times for 5 min and rehydrated twice in fresh 100% ethanol, 95% ethanol, 70% ethanol, and 50% ethanol for 3 min each. Sections were washed in double distilled H_2O and permeabilized with 0.1% Triton X-100 in 1X PBS. Heat induced antigen retrieval (HIER) was performed by heating sections in 1 mM EDTA at 95°C in a pressure cooker for 20 min followed by 20 min of cooling at RT. Sections were incubated for 12 h at 4°C in blocking buffer (5% goat serum + 0.3% triton x-100 + 3% BSA diluted in 1 x PBS) and subsequently incubated with mouse anti-dsRNA J2 antibody, diluted 1:200 in blocking buffer, for 2 h at RT. Sections were washed in 0.1% Triton X-100 in 1X PBS three times for 10 min and incubated with secondary antibody goat anti-mouse Alexa Fluor 488 at 1:200 for 1 h at RT and washed again with 1X PBST. All processed slides were mounted in Prolong gold antifade mount with DAPI (Invitrogen cat # P36931). Some slides where processed in the absence of primary antibody to verify specificity of labelling.

Imaging of immunohistochemistry samples

All images were obtained with a 100X objective on a Leica TCS-SP8 inverted spectral confocal microscope (Leica Microsystems: Mannheim, Germany) equipped with a 405 nm blue diode laser, argon laser (5 lines), and white light laser for excitation. Further image processing of maximal *z*-projection images of 4 μM thick liver sections showing 488 and DAPI overlay was performed on LAS X version 3.30 software. Identical settings were used to obtain fluorescent images within data sets. Brightness and contrast for final images were adjusted equally across data sets using Photoshop CC 2017. Confocal laser scanning microscopy was performed at the CNSI Advanced Light Microscopy/Spectroscopy Shared Resource Facility at UCLA.

Quantification of immunohistochemistry samples

To quantify J2 dsRNA immunofluorescence in liver sections from WT C57BL/6 and *Pnpt1*^{HepKO} (HepKO) mice, a single in-focus plane was acquired at 100x at 20-21 locations across the tissue selected using a random coordinate generator. Quantifications were performed using ImageJ software, by drawing an outline around tissue and measuring area, integrated density, and mean fluorescence. Additionally, background readings were measured on 2nd only tissue samples. To calculate the corrected total fluorescence intensity (CTFI) we used the following formula: CTFI = integrated density – (area of selected tissue × mean fluorescence of background readings). Scatter plot and statistical analysis (2-sided unpaired t tests with Welch's correction) were performed using GraphPad Prism 7.

Flow cytometry analysis

Cells were trypsinized, washed with PBS and fixed with 4% formaldehyde diluted in PBS for 20 min RT. Cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min followed by incubation in 1% BSA (Sigma, A7030) in PBS for 1 h. Primary antibodies anti-dsRNA (J2) or normal mouse IgG2a (Iso) was used at 2.5 µg/ml in 1% BSA for 1 h at RT. Secondary donkey anti mouse IgG (H+L) conjugated with Alexa Fluor 488 were used at 2.2 µg/ml concentration for 1 h at RT. Cells were rinsed 3X with FACS buffer (0.5% BSA in PBS with 2mM EDTA). Cells were acquired with a FACSCalibur™ (BD Biosciences) flow cytometer. Data was analyzed in FlowJo (TreeStar).

Immunoprecipitation of dsRNA

Protein G Dyna beads were washed and resuspended in NET-2 buffer. 5µg of anti-dsRNA mAb (J2) was bound to 100 µl of beads for 1 h at RT on a Thermoshaker. Conjugated beads were washed thrice with NET-2 Buffer. 80-90% confluent HeLa cells from 10 cm^2 plate (x2) were washed with 10 mL of cold PBS. Cells were scraped and transferred to a falcon and spun at 500 xg at 4°C, 5 min. Cell pellet from one 10 cm^2 plate was lysed in 1 ml of NP-40 lysis buffer and transferred to an eppendorf and incubated on ice for 5 min. Following a spin at 13000 RPM at 4^{0}C, 5 min supernatant was carefully transferred to a new eppendorf. Total RNA was harvested from 10% input lysate using Trizol reagent. For IP, lysate was diluted 1:4 in NET-2 buffer and supplemented with 10 units of RNase free TurboDNase (Ambion) at 10 mM $MgCl_2$ per 1 ml of mix. 100 µl of J2-dynabeads was added to 1 ml of above lysate and left for 1-2 h at 4^{0}C. Following magnetic separation, beads were washed twice with 1 ml of high salt washing buffer (HSWB). Beads were transferred to a new tube with NET-2 buffer and washed twice with the same buffer. J2-bound dsRNA was extracted with Trizol reagent. The RNA samples were sent for sequencing described in "RNA-sequencing" Method section. NET-2 buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% NP-40), NP-40 lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40), High Salt Wash buffer (50 mM Tris-Cl pH 7.4, 1M NaCl, 1mM EDTA, 1% NP-40, 0.5% DOC, 0.1% SDS).

dsRNA isolation for northern blot.

293 cells from 150 cm^2 plate (x3) were used. Cell pellet was lysed in 4.5 mL of NP-40 lysis buffer and kept on ice for 5 min. Lysate was transferred to 1.5 mL eppendorf and spun 20000x g at 4^{0}C for 5 min. The supernatant was then transferred to 15 mL tube. Lysate was diluted 1:4 in NET-2 buffer and supplemented with 12 units of RNase free TurboDNase (Ambion) and 10 mM $MgCl_2$ per 1mL of final mix. RNases were added (RNase T1-1U from 1U/µl,

RNase V1-1U from 0.1U/µl (LifeTechnologies) and incubated at 37°C for 10 min. 100 µl of J2-dynabeads were added to 1 ml of above lysate and left on a rotor at 4^0C for 1-2 h. Beads were spun at 3000 g at 4°C, 3 min. Supernatant was discarded and beads transferred to 1.5 mL tube, washed twice with 1 ml of HSWB and washed twice with NET-2 buffer. J2-bound dsRNA was extracted with Trizol reagent. RNA samples were used for northern blot.

Northern blot analysis

dsRNA after immunoprecipitation was purified by TRI Reagent (Sigma) using the manufacturer's protocol. 20% of dsRNA eluate was dissolved in denaturing solution and run on a 1% agarose gel as described previously [12]. Subsequently, RNA was transferred to Amersham Hybond-N+ membrane (GE Healthcare Life Sciences) and UV cross-linked. For detection of mitochondrial transcripts probes were labeled with [α-^{32}P] dATP (Hartmann Analytic) using a DECAprime™ II Kit (Ambion). PCR products corresponding to the following fragments of human mtDNA were used as templates: 254–4469 (Probe 1), 4470–8365 (Probe 2), 8365–12137 (Probe 3), 12091–16024 (Probe 4). Hybridizations were performed in PerfectHyb Plus buffer (Sigma) at 65°C. Membranes were exposed to PhosphorImager screens (FujiFilm), which were scanned following exposure by a Typhoon FLA 9000 scanner (GE Healthcare). Data were analyzed by Multi Gauge V3.0 software (FujiFilm).

Probe for RNA protection assay (RPA)

U1 snRNA antisense fragment was amplified from pGEM4-tU1 (Shona Murphy, Oxford) by PCR using the following primers. tU1_F: AGCTCGGATCCATACTTACCTGGCAGGGGAGATAtU1_R: ATTCATTAATGCAGCTGGCTT According to the manual of StrataClone Blunt PCR cloning kit (Agilent genomics), the PCR product was cloned as pSC-B-tU1_RPA. T7 transcription was performed using [γ-^{32}P] UTP and XhoI-digested pSC-B-tU1_RPA to label the antisense tU1 RNA. The radio labelled RNA was purified from denaturing gel.

***In vitro* J2 immunoprecipitation (IP) assay**

In brief, 2000 cps ^{32}P labelled antisense tU1 RNA was incubated with 10 µg of purified HeLa nuclear RNAs followed by RNAse protection analysis (RPA) [31]. After RPA, dsRNA was IPed with 5 µg of J2 antibody conjugated protein G beads (Thermo Fisher Scientific) in NET-2 buffer. ^{32}P labelled antisense tU1 RNA was used as ssRNA substrate. The antibody beads were washed with NET-2 buffer several times and then incubated with Trizol (Thermo Fisher Scientific) to purify IPed RNAs. The RNAs were analyzed on 8% denaturing gel.

MtRNA isolation and treatment of cells

Mitochondria were isolated from HeLa cells by using magnetic cell separation procedure as described by the manufacturer (Mitochondria Isolation Kit; MACS, Miltenyl Biotec). RNA was purified from mitochondria using Trizol reagent (Sigma) and was DNaseI treated with Turbo DNase. 1 µg of mtRNA was transfected onto HeLa cells and 300 ng in case of MEFs in 1:3 ratio of lipofectamine-2000 in 12 well plates with cells at 80% confluency. For enzymatic treatment, 1 µg of mtRNA was incubated with RNase III as per manufacturer's instructions. 100 ng of

ppp-IVT-RNA^{99nt} and CIP-EMCV-RNA were transfected in MEFs in 12 well plates using lipofectamine-2000. Total RNA from HeLa cells/MEFs was extracted 20 h post transfection for IFN-β or *Ifit1* mRNA quantification respectively. In Extended Data Figure 6b, HeLa cells 65h post siRNA transfection were treated with ABT-737 (10 μM) or DMSO and incubated further for 8 h. Total RNA was isolated using Trizol for IFN-β mRNA quantification.

Separation of Cytoplasm and Mitochondria fractions

Mitochondrial and cytoplasmic fractions in Extended Data Figure 6c were prepared as previously described[32]. Purity of fractions was tested by western blot.

Detection of mtRNA in cytosolic extracts.

Cytosolic extracts were prepared using digitonin extraction as described previously[8]. Digitonin (sigma) at 25 μg/ml was used for Patient fibroblast cells. qRT-PCR was performed on RNA isolated from cytosolic fractions using random hexamers for cDNA synthesis followed by PCR using mtDNA specific primers (Extended Data Table 2) normalized to β-actin mRNA levels.

RNA-sequencing

RNA-seq was performed by the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics, University of Oxford. Input RNA samples were ribo-depleted with Ribo-Zero rRNA-removal kit (Human/Mouse/Rat, EpiCentre RZH110424). Immunoprecipitated RNA samples were not ribo-depleted. Libraries were prepared with the NEBNext Ultra Directional RNA Library Prep Kit for Illumina, v1.0 (cat. no. E7420) according to the manufacturer's guidelines. Libraries were sequenced on an Illumina HiSeq-2000 with 100-bp paired-end reads, v3 chemistry.

Microarray method

Hepatocytes were isolated from perfused livers of two PNPASE (*Pnpt1*) liver specific knockout C57BL/6 mice (HepKO; 1 male aged 12.9 weeks, 1 female aged 4.29 weeks, two independent experiments) and two sex-matched wildtype littermate mice (WT). Total RNA was extracted from hepatocytes using TriZol Reagent (Invitrogen Cat. #15596026), followed by the Qiagen RNeasy Mini Kit (Qiagen, Cat. # 74104). Labeled cRNA was generated using 200 ng of total RNA from each sample and the Agilent Low RNA Input Linear Amplification labeling kit. Each labeled sample was hybridized against its gender-matched sample in fluor-reversed pairs of arrays to an Agilent 4x44k Mouse Whole Genome Microarray. The arrays were scanned using the Agilent DNA Microarray Scanner, and data was extracted using the Agilent Feature Extraction (version 9.5.1.1) software using the standard Agilent protocol except without Lowess normalization. The fluor-reversed pairs were combined into Experiments in Rosetta Resolver 7.1 to produce the male and female signature gene ratios. We performed age and gender-matched differential expression analysis and generated a list of signature genes with significant differential expression in both the male and female cohorts. Both male and female cohorts showed very similar results. For simplicity, only fold expression changes in female mice were shown.

IFNreactome methods

The gene set for interferon signaling (encompassing IFN a/b/g signaling and IFN stimulated antiviral response) was extracted from the Reactome database under pathway ID R-MMU-913531[33]. An additional antiviral innate immune response gene set was curated from recent studies investigating the role of mitochondrial DNA in innate immunity[8]. Corresponding genes from the set were compared to the HepKO signature genes (adjusted *P* value < 0.05, significant in both female and male matched pairs). Overlapping genes were plotted using the male and female matched $\log_2$ (Fold Change). Both male and female data showed very similar results. For simplicity, only fold expression change data from female mice were shown.

Immuno-electron microscopy

HeLa cells were grown in a 6 well plate and siRNA treated for 65 h, trypsinised and pelleted for 1 min at 3000 rpm in cell culture media containing 5% FBS and 20% BSA. Cells were then immediately cryo-fixed using a Leica EM PACT2 high pressure freezer and then further processed as described[34], except that tannic acid was omitted from the freeze substitution medium. Blocks were sectioned on a Leica UC7 ultramicrotome using a diamond knife (Diamtome). 90 nm sections were transferred to 200 mesh Nickel grids and then immunolabelled as follows: Grids were floated section side down on a 20 µl droplet of blocking solution (10% goat serum in TBS) for 15 min, then blotted and incubated on a droplet of primary antibody (diluted 1:25 in buffer A – 1% BSA, 1% goat serum, 0.01% Tween-20 in TBS) for 2 h at RT. Grids were washed by passing them over 5 droplets of buffer A, 5 min each, then incubated with secondary antibody (Abcam goat anti-mouse conjugated to 20 nm gold) diluted 1:10 in buffer A for 90 min at RT, then washed by passing over 3 droplets of water. Sections were then post-stained for 10 min with uranyl acetate and Reynold's lead citrate and imaged as described above.

Statistical analysis

Unless otherwise stated, the figures present the average values of at least three independent experiments mean ± SD or s.e.m. For analyses with n>10, individual data points are shown. The mean is reported when n=2, and no other statistics were calculated for these experiments.

Bioinformatics: Mapping of sequencing reads and data visualization

Paired-end reads for each sample were mapped to the human genome reference assembly GRh37/hg19 (build 37.2, Feb 2009) using the Bowtie2 alignment software[35]**.** Prior to alignment, adaptor sequences were trimmed using Cutadapt 1.8.3, discarding reads with less than 10 bases. An in-house Perl script was used to remove the reads left unpaired (code available upon request). SAMtools 1.2[36] was used to process aligned reads to only include uniquely mapped reads with no more than two mismatches. Data were scaled to library size (genomeCoverageBed) using Bedtools[37]. Bigwig track files were generated from the Bowtie2 output files using UCSC bedGraphToBigWig tool[38]**.** Correlations among different samples for chrM were calculated with R. Data from replicates (n=2 for each condition) except where untreated and sicntrl treated samples (r=0.85) were then merged and viewed on the UCSC genome browser (Extended Data Figure 5a). For chromosome-wise read

coverage plot, number of filtered reads mapping for each chromosome were counted. These numbers were then normalised to the size of respective chromosome. For plotting the distribution of reads belonging to different RNA species, reads mapped to each Ensembl biotype annotations were counted for using bedtools and then normalized to the size of the genome. Heatmap was generated with the R-package gplots using a subset of significantly altered interferon simulated genes identified by gene expression analysis on total RNA-seq from siLuc (control siRNA) and two knockdown conditions (siSUV3 and siPNPase).

To screen for RNA editing candidates in chrM, REDItoolDenovo.py script that is part of REDItools package[39] was used. To avoid the risk of using unreliable editing sites, the output of REDItools was passed through two stringent filters. Firstly, all editing sites were removed that had less than 10 edited counts and total read coverage <50 for all the knockdown libraries. Secondly, only those editing sites were considered for siSUV3, siPNPase where the frequency of editing was at least 1.5 fold to that of siCntrl. The resulting editing sites were then filtered for known SNPs in the mitochondrial transcriptome[40] to obtain reliable RNA editing candidates.

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Data availability

GEO accession numbers: GSE94957 and GSE109210 (mouse microarrays). Source Data for graphical representations obtained from PNPase KO mice microarray are provided in the Supplementary Information of this manuscript. Electrophoretic gel Source Data are presented in Supplementary Figure 1.

Extended Data Figure 1. Characterization of anti-dsRNA J2 antibody and mtDNA depletion results in loss of mtdsRNA formation.

a, qRT-PCR analysis of L-mRNA expression in encephalomyocarditis virus (EMCV) infected HeLa cells at a multiplicity of infection (MOI) of 1 at indicated time points post infection (n=2 independent experiments). **b**, confocal microscopy images of uninfected or EMCV infected HeLa cells (MOI 1), 8 h post infection stained with anti-dsRNA antibody (J2) and nuclear stained with DAPI (representative of two experiments). Scale bars represent 10 µm. **c**, immunostaining of dsRNA and DNA in HeLa cells treated with indicated nucleases before staining. Signal from J2ab is specific for RNA but not for DNA and is sensitive only to RNase III treatment (representative of three experiments). Scale bars represent 10 µm. **d**, quantitation of fluorescence signal from HeLa cells treated as in panel c. Mean ± s.e.m., n=4095, 1755, 4766, 5585 respectively. **e**, autoradiogram showing substrate specificity of J2 based on IP efficiency for uniformly ^{32}P-radiolabelled ssRNA and dsRNA substrates. Signals were visualized and quantified by PhosphorImager. The level of IP signal is shown and expressed as percentage of input (representative of two experiments). For gel source data, see Supplementary Figure 1. **f**, chromosome-wide coverage plot of dsRNA-seq reads. Inset pie chart illustrates read distribution of dsRNA-seq based on RNA class biotypes depicted by different colours. **g**, dsRNA and DNA staining of HeLa cells transfected with constructs encoding indicated proteins, the expression of which results in mtDNA depletion. Plasmids encoding mtDNA-depletion factors co-express EGFP from an independent promoter, which enables identification of transfected cells. Mitochondria were stained using anti-OXA1L antibody. Scale bars represent 10 µm. Lower panel represents quantitative analysis of fluorescence signal from HeLa cells in the above experiment. Mean ± s.e.m., n=10 cells.

Extended Data Figure 2. RNA degradosome components SUV3 and PNPase are involved in mtdsRNA turnover.

a, HeLa cells treated with DMSO, DRB (100 µM) and actinomycin-D (0.5 µg/ml) for 60 min and stained with anti-dsRNA (J2) ab. Mitochondria were stained with MitoTracker Red CMXRos (representative of two experiments). **b**, flow cytometric analysis of dsRNA levels in HeLa cells treated with indicated siRNAs. Cells were labelled with J2 ab or isotype control (Iso). Mean ± SD (n=3 independent experiments). **c,** detection of dsRNA with J2ab in HeLa cells after depletion of PNPase or SUV3 by On-TARGETplus siRNAs (indicated with an asterisk and listed in Extended Data Table 2). Mitochondria were stained with MitoTracker. Scale bars 10 µm. Western blot showing PNPase or SUV3 depletion (representative of four experiments). For gel source data, see Supplementary Figure 1. Quantitation of dsRNA levels in HeLa cells depleted of PNPase or SUV3. Mean ± SD (n=4 independent experiments). **d**, quantitative analysis of fluorescent signals from dsRNA in HeLa cells with depleted enzymes involved in mitochondrial nucleic acids metabolism. Mean ± SD (n=4 independent experiments). **e**, HeLa cells were transfected with siRNA specific for PNPase, SUV3, or non-targeting control. Prior to

fixation, cells were treated for indicated times with inhibitors of transcription: actinomycin-D (0.5 μg/ml), DRB (100 μM). Immunostaining of dsRNA was performed and cells were imaged by a fluorescent microscope screening station. Mean ± SD (n=4 independent experiments).

Extended Data Figure 3. Unwinding activity of SUV3 is required to suppress mtdsRNA accumulation.

a, confocal images of 293 cells expressing stably integrated wild type SUV3 (hSUV3_WT) and catalytically inactive (G207V) dominant negative form (hSUV3_G207V) stained with J2 ab (green). Mitochondria stained with MitoTracker deep Red (red). Nuclei stained with Hoechst 33342 (grey). Right panel represents quantitative analysis of fluorescence signal from HeLa cells in the above experiment. Mean ± s.e.m., n=16 cells. **b**, northern blots of J2 IPed dsRNA from hSUV3_WT and hSUV3_G207V overexpressing 293 cell lines with four different probes spanning entire mitochondrial genome. Lower diagram depicts positions of probes on mitochondrial genome (representative of two experiments). For gel source data, see Supplementary Figure 1.

Extended Data Figure 4. Exonuclease activity of PNPase is required to suppress mtdsRNA formation.

a, diagram of PNPase domain structure showing position of R445E/R446E mutation in RNasePH domain. **b**, immunostaining of dsRNA in HeLa stable cell lines transfected with siRNA specific for PNPase or non-targeting siRNA (control). Depletion of endogenous PNPase was rescued by expression of siRNA-resistant PNPase-FLAG protein. Wild-type or mutated, RNA-degradation deficient version of PNPase was expressed (R445E/R446E). Mitochondria stained with MitoTracker. Scale bars 20 μm. **c,** western blot analysis of PNPase in HeLa cells treated as in panel b. Exogenous, siRNA-resistant PNPase is expressed as FLAG fusion (representative of three experiments). **d,** quantitative analysis of fluorescent signals from dsRNA in HeLa treated as in panel b. Mean ± SD (n=3 independent experiments). For gel source data, see Supplementary Figure 1.

Extended Data Figure 5. DsRNA-seq of HeLa cells following siRNA depletion of SUV3 and PNPase .

a, dsRNA-seq reads across the mitochondrial genome spanning all protein coding regions (~3.5-15.5 kb) following siRNA treatment (n=2 independent experiments). Blue coverage denotes H-strand and red L-strand reads. Short bars depict tRNA and long bars mRNA. **b**, correlation plots of J2 IP dsRNA-seq replicates. Pearson correlation coefficients are calculated and shown on each plot.

Extended Data Figure 6. Upregulation of ISGs in HeLa and murine cells following loss of PNPase accentuated by mitochondrial outer membrane permeabilization.

a, heat map of interferon stimulated genes (ISGs) generated from a subset extracted from list of significantly expressed genes. Gene expression is depicted by colour intensity. Green denotes upregulation and red downregulation. **b**, qRT-PCR analysis of IFN-β mRNA in HeLa cells treated with indicated siRNAs after 8 h of treatment with vehicle or ABT-737 (Mean (n=2 independent experiments). **c**, western blot analysis of the cytochrome c release into the cytoplasm of HeLa cells treated with ABT-737 for 8 h. Subcellular fractionation purity confirmed by relevant markers. (representative of two experiments) **d**, log2 fold change expression levels of ISGs and genes involved in IFN signaling in *Pnpt1*^{HepKO} (HepKO) vs WT female mice. ISG list is based on the Reactome database[33].

e, western blot of whole cell extracts from indicated siRNA treated cells (representative of two experiments). For gel source data, see Supplementary Figure 1.

Extended Data Figure 7. RNA editing of cytoplasmic mtRNA.

a, RNA editing sites mapped on the RNA transcriptome of SUV3 and PNPase depleted cells are shown. No editing sites were observed in siCntrl. Each dot represents an editing event. Dots on the upper panel denote editing events on the H-strand and dots on the lower panel denotes editing on the L-strand. Yellow bars denote the D-loop region. **b**, frequency of dinucleotide RNA editing sites mapped in the PNPase depleted samples. **c**, qRT-PCR analysis of IFN-β mRNA levels in indicated siRNAs treated cells (Mean (n=2 independent experiments) **d**, western blot of ADAR1, SUV3 and PNPase in indicated siRNA treated cells (representative of two experiments). For gel source data, see Supplementary Figure 1.

Extended Data Figure 8. EMCV infection results in dsRNA accumulation partially overlapped with mitochondria.

a, EMCV infected HeLa cells at multiplicity of infection (MOI) 1, 8 h post infection stained with anti-dsRNA J2 ab (dsRNA) and nuclear stained with DAPI. Line scan RGB profile for the region of interest (ROI) selected with a white line is shown on the right (representative of two experiments). **b**, zoomed in view of ROI of EMCV infected HeLa cell showing colocalization of dsRNA with mitochondria (representative of two experiments). Scale bars 10 μm.

Extended Data Table 1: Clinical table of patients carrying PNPT1 mutations

CSF=cerebrospinal fluid; het=heterozygous; hets=heterozygotes; hom=homozygous; RC=respiratory chain; NA= not available; ND= not determined; *Normal value is <2.466.

Extended Data Table 2: Oligonucleotide primers and siRNAs used in the study